PB85-153526

Electrophysiological Detection of Sublethal Neurotoxic Effects in Intact Earthworms

Iowa State Univ., Ames

Prepared for

Corvallis Environmental Research Lab., OR

Jan 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB85-153526

EPA/600/D-85-002
January 1985

ELECTROPHYSIOLOGICAL DETECTION OF SUBLETHAL NEUROTOXIC EFFECTS IN INTACT EARTHWORMS

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EPA Cooperative Agreement CR8100060-02

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OFFICE OF RESEARCH AND DEVELOPMENT
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TECHNICAL REPORT DATA *(Please read Instructions on the reverse before completing)*		
1. REPORT NO. EPA/600/D-85/002	2.	3. RECIPIENT'S ACCESSION NO. PB85 153526/AS
4. TITLE AND SUBTITLE Electrophysiological Detection of Sublethal Neurotoxic Effects in Intact Earthworms		5. REPORT DATE January 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Charles D. Drewes, Elizabeth P. Vining, and Clarence A. Callahan		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Zoology Iowa State University Ames, Iowa 50011		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO. CR8100060-02
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Corvallis, Oregon 97333		13. TYPE OF REPORT AND PERIOD COVERED Published Paper 14. SPONSORING AGENCY CODE ORD/600/02
15. SUPPLEMENTARY NOTES		

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17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS *(This Report)* Unclassified 20. SECURITY CLASS *(This page)* Unclassified	21. NO. OF PAGES 27 22. PRICE

EPA Form 2220-1 (9-73)

NOTICE

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ABSTRACT

The nervous system of earthworms is a logical site for studying the deleterious effects of many toxic substances because neural functions are often more sensitive to disruption than other physiological processes. However, the sublethal effects of toxicants on neural and behavioral functions may be subtle and their detection and quantification may require use of relatively sophisticated electrophysiological testing methods. Recently, we have developed methods for non-invasively recording the bioelectrical events accompanying rapid escape reflexes in intact earthworms using printed circuit board grids. These methods permit assessment of the sublethal effects of various toxicants on nervous system function and motor activity in a wide variety of earthworm species of all ages.

Results are described in which non-invasive recording procedures were used in combination with several different laboratory protocols for toxicant exposure. These included 1) 48 hour contact exposure, 2) two week artificial soil exposure, 3) immersion exposure, and 4) long-term growth studies in treated soil mixtures. The results indicate that non-invasive electrophysiological testing of escape reflex function can be used as a sensitive means of detecting sublethal neurophysiological impairment by pesticides and other environmental pollutants. Further study is now needed to determine if comparable impairment of neural and motor function occurs in chemically-treated animals in the field. If so, then such impairment would likely disrupt ecologically significant locomotory activities of the worms, including escape from predators or burrowing activities within the soil.

INTRODUCTION

Earthworms are continually exposed to and accumulate a wide variety of pesticides and other toxic substances (Edwards, 1966; 1973a,b; Gish, 1970; Edwards and Thompson, 1973; Thompson, 1973; Gish and Hughes, 1982). One of the most common approaches to assessing possible adverse biological effects of these toxicants is determination of mortality (LC_{50}) following treatments with known dosages of chemicals. However, many soil contaminants are not highly lethal to earthworms (Davey, 1963; Edwards, 1979; Thompson and Edwards, 1974), and therefore it seems likely that the sublethal effects of the contaminants (e.g., effects on growth, reproduction, or behavior) may be much more critical in diminishing natural populations (Thompson, 1973). The nervous system of earthworms is a logical site for investigating such sublethal effects because 1) it controls behavior and many other biological processes, and 2) many contaminants are primarily neurotoxic.

During the past few years we have been involved in developing sensitive and reliable methods for quantifying sublethal neurotoxic effects in earthworms. To do this we selected one biologically important reflex behavior, the worm's rapid escape response, and developed a means of non-invasively assessing alterations in the bioelectrical events underlying the response. The reflex response consists of a rapid longitudinal shortening triggered by various threatening stimuli, such as substrate vibration or touch. The escape reflex is particularly conducive to such assessments because it is mediated by giant nerve fibers which generate large, rapidly conducted impulses that are easily detected with electrodes placed along the ventral surface of the worm (Drewes, 1984). The reflex itself is important from an ecological standpoint because it represents a fundamental means for escaping from potential predators. It is our hope that this reflex may be used for ecotoxicological assessment in much the same way that clinicians use

spinal reflexes in animals and humans for electro-diagnosis of neurotoxicity or neuropathology.

The techniques we have developed provide a number of experimental advantages. These include:

(1) the sensitivity of the method, which allows quantification of sublethal neurotoxic effects which might otherwise be difficult to quantify in observations of overt behavior,

(2) no requirements for anesthesia or immobilization,

(3) capabilities for repeated testing of the same worms throughout long-term or chronic exposure,

(4) capabilities for assessing neurotoxicity in all ages (including embryonic stages) and virtually all species of earthworms, and

(5) capabilities for simultaneously assessing disruptions in central as well as peripheral nervous system function in different body regions.

Other factors to consider are the relatively low cost of implementing the recording system (less than $10,000) and the relatively short time required for training personnel to effectively utilize the technique. If necessary, the recording set-up could be made portable for rapid assessment of neurotoxicity in freshly collected worms at or near field sites.

ELECTROPHYSIOLOGICAL TESTING PROCEDURES

A variety of recording grids can be used for detection of electrical impulses in the giant nerve fibers of earthworms (Drewes et al., 1978; Balter et al., 1980). However, for toxicological purposes the preferred system consists of an array of parallel, metalic grid lines etched onto a printed circuit board (for details, see O'Gara et al., 1982). The overall size of these grids, as well as electrode spacings, can be varied in accordance with the dimensions of the worms. Spacing between grid lines for embryonic worms may be as small as 250 µm (O'Gara et al., 1982), or for large adult specimens

up to several mm (Drewes et al., 1983). Figure 1 shows an earthworm positioned with its ventral surface in contact with four pairs of recording electrodes. Signals from the electrode pairs are fed into differential recording amplifiers, filtered, and displayed as multiple traces on the storage oscilloscope (Drewes and McFall, 1980; Drewes et al., 1984). For most species of worms impulse amplitudes range from approximately 20-100 μV, with signal-to-noise ratios varying from about 2:1 to 10:1.

A number of devices can be used to stimulate giant nerve fiber impulse activity. For tactile stimulation, a light and focal touch with a small hand-held stimulus probe is delivered to some portion of the body, usually the head or tail. Mechanical contact of the animal with the tip of the probe can be used to make or break a simple electrical circuit, thus providing a transient electrical signal which is used to trigger a single sweep of the oscilloscope (Drewes and McFall, 1980; O'Gara et al., 1982). An alternative is to glue a light weight extension onto the needle of a phonograph cartridge. When the animal is touched with the tip of the extension, the transient output of the cartridge is sufficient to trigger the oscilloscope sweep (Vining and Drewes, 1984). For the purpose of producing a precisely controlled tactile stimulus, a light weight rod can be glued to the diaphragm of a small speaker. The speaker is then mounted on a micromanipulator and driven by electrical pulses from a square-pulse stimulator (Drewes and Vining, 1984). The production of precisely controlled stimuli is particularly desirable for assessing changes in touch sensitivity of escape reflexes following toxicological treatment.

Since some toxicants could selectively block touch sensory pathways, it may be desirable to test for giant nerve fiber excitability by circumventing sensory pathways. In this case electrical stimuli, delivered via the grid,

can be used for direct electrical excitation of giant fibers (Vining and Drewes, 1984).

Regardless of the specific mode of giant fiber stimulation, giant fiber responses in normal animals can be reliably evoked during many minutes or hours of repeated testing. If stimulus strengths are just adequate for evoking one or two giant fiber spikes, muscle responses are small and animals make little or no attempt to crawl away. On the other hand, use of stimulus strengths which evoke high frequency trains of giant fiber spikes will often result in vigorous withdrawal and crawling responses, thus displacing the animal from the recording sites and delaying subsequent testing. Worms which have crawled away from the recording sites can be readily coaxed to crawl back into position by gently stroking head or tail segments with a fire-polished glass rod.

Figure 1 shows an oscilloscope recording of one lateral giant nerve fiber impulse recorded from an adult specimen of Lumbricus terrestris. The figure illustrates how conduction velocity values are determined from such records. Several precautions should be taken when obtaining representative conduction velocity values:

(1) a reliable mean is obtained from three to six replicate measurements;

(2) replicate measurements should be made within the same body region, since velocities systematically vary in different portions of the same animal (O'Gara et al., 1982);

(3) measurements should be made using the first impulse in a train of impulses, since conduction velocity is faster for the second and later spikes in a high-frequency train (Drewes et al., 1978);

(4) the site of stimulation should always be well anterior or posterior to all recording sites;

(5) measurements should be made only when worms are oriented in straight line and in a reasonably elongated state; and

(6) all measurements should be made at a constant temperature.

If these precautions are met, the intra-animal variability in lateral and medial giant fiber conduction velocities is very low when comparing means from the same animal on successive days. In fact, given a small group of adult worms, the combination of medial and lateral giant fiber velocities from various body regions is sufficiently unique that each animal can be unequivocally distinguished from the other. Thus velocity values together with other characteristics, such as sensory field boundaries for the giant fibers, may be used as a type of neurophysiological "fingerprint" for each animal (Drewes et al., 1983).

Mean velocities are determined at various times during exposure, or at the termination of exposure, and compared to original values. Pre-treatment and post-treatment values may be represented either as absolute velocities (e.g. see Fig. 6) or as relative velocities (e.g., see Figs. 2, 3 & 4). Relative velocity is defined as the ratio of the mean velocity at a particular time after treatment to mean velocity before treatment (Drewes and Vining, 1984). For a group of worms the mean relative velocity after treatment can then be compared to the initial relative velocity value (i.e., 1.0) using a t-test for related measures.

CONTACT EXPOSURE

This procedure involves exposure of individual worms to an aqueous solution or suspension of the toxicant (Goats and Edwards, 1981; Drewes and Vining, 1984). A single animal is placed within a closed vial which is lined with appropriately shaped pieces of filter paper. Stock solutions and serial dilutions of the chemical are prepared in acetone, and an aliquot of the solution is pipetted into each vial. The acetone is then evaporated leaving a

relatively uniform distribution of the chemical on the paper. Next an aliquot of water is pipetted into the vial, thus saturating the paper. After pretesting, the worm is placed in the vial for a prescribed time and retested. Concentrations in the vial can be expressed as parts of chemical per million parts of water (ppm) or µg chemical per cm^2 of paper (Goats and Edwards, 1982). In control groups, the vials are lined with filter paper and treated with acetone. After evaporation of the acetone, an aliquot of water and a worm are placed in each vial. Worms treated in this way survive for many days without statistically significant changes in neurophysiological parameters (Drewes and Vining, 1984).

Examples of results obtained following 48 h contact exposure of adult Eisenia foetida to dieldrin are shown in Figure. 2. The results indicate giant fiber conduction velocities were significantly decreased at concentrations as low as 1-10 ppm. The reduction was dose-dependent, with relative velocity declining to approximately 0.65 (medial giant fiber) and 0.85 (lateral giant fiber) at 288 ppm. By comparison the LC_{50} value using identical exposure testing was at least three orders of magnitude greater than the threshold concentration for neurotoxic effects (LC_{50} = 8100 ppm; 95% CI = 4500 - 14,500 ppm; Drewes et al., 1984).

These results clearly demonstrate that contact exposure, in conjunction with non-invasive neurophysiological testing, can be used as a sensitive means of detecting sublethal toxic effects. Based on contact exposures to a wide variety of other pesticides and toxic compounds, it appears that comparable sublethal reductions in conduction velocity often occur. Although the sensitivity for detecting dieldrin effects is especially high, effects of other toxicants are usually evident at concentrations ranging from one or two order of magnitude below lethal levels. In unusual cases, such as contact exposure to dimethyl phthalate (a plasticizing agent and insect repellent

ingredient), no sublethal effects on conduction velocity were apparent after 48 h (Drewes et al., 1984).

ARTIFICIAL SOIL EXPOSURE

Chronic exposure to chemicals, within a context more similar to soil environment, can be achieved using artificial soil exposure (Goats and Edwards, 1981). The dry constituents of artificial soil include fine sand, kaolin, and peat, which are mixed together in a percentage weight ratio of 70:20:10. If insoluble in water, the test chemical can be dissolved at a known concentration in an acetone/water solution, and mixed into the dry artificial soil mixture. The acetone and water are then evaporated off, leaving a relatively uniform distribution of the chemical in the soil. Then water is added to rehydrate the mixture; we routinely use 50 g water per 100 g dry mixture). The hydrated mixture can then be apportioned to completely fill individual containers, such as glass petri dishes. Chemical concentrations in the containers can be expressed as mg of chemical per kg of dry soil (= ppm). Control groups are treated identically, except the test chemical is not included in the mixture.

Some representative results obtained following a two-week exposure to dimethyl phthalate (DMP) are shown in Figure 3. The results show that DMP caused sublethal and statistically significant reductions in conduction velocity in both medial and lateral giant nerve fibers. No significant reductions in velocity were evident in control groups (0 ppm). Apart from the DMP-induced changes in conduction, there were no other apparent behavior or neurophysiological effects. Presumably, these changes were a consequence of prolonged exposure, since no DMP-induced reductions in conduction velocity were evident in 48 h contact tests (Drewes et al., 1984).

IMMERSION EXPOSURE

Most earthworms can naturally withstand complete submersion in water for long periods of time, thus providing another possible mode of exposure for toxicity studies. In contrast to contact and artificial exposure, immersion exposure in chemically treated water provides a means of studying toxicity under conditions of nearly maximal surface area for exposure and optimal uptake (Lord et al., 1980). For the purpose of rapidly "loading" an animal with a toxicant, a carrier solvent, such as dimethysulfoxide (DMSO), can be mixed with water. In these cases neurotoxic effects of the chemical can occur after exposure for only a few minutes.

Figure 4 shows results obtained with adult earthworms (Lumbricus terrestris) immersed for 2 min in an aqueous solution (10% DMSO) of the organophosphate pesticide, Diazinon (Ciba-Geigy). After immersion, animals were rinsed for 1 min in water and then tested for changes in conduction velocity. The results show a dose-dependent reduction in velocity within 15 min after exposure. Worms exposed at the highest concentration were dead by 2 h after exposure. In addition to decreases in conduction velocity, other neurophysiological symptoms were evident. These included a gradual loss of the motor neuron and longitudinal muscle electrical responses that normally accompany medial giant nerve fiber impulse activity (c.f. Drewes et al., 1980). As shown in Fig. 4, many of the animals achieved full recovery from the treatment, although the speed of recovery appeared inversely related to the chemical concentration.

GROWTH EFFECTS

Although some of the previously described exposure protocols can be used for long-term chemical exposure, none provides a food source. The provision of a food source, in combination with chemical treatment, raises the possibility of studying treatment-related effects on somatic as well as nervous system growth and development.

We have recently studied the effects of a metal toxicant, cadmium, on somatic and nervous system growth in Eisenia foetida raised individually in a chemically treated mixture of garden soil and horse manure. To prepare the mixture the chemical ($CdCl_2$) was dissolved in a small volume of water and atomized onto and thoroughly mixed with a known quantity of thawed horse manure (75% water by weight). After mixing, 40 g of moistened soil (25% water by weight) was mixed with 20 g of the treated, moist manure and placed in a petri dish along with a newly hatched worm. Animals were regularly weighed and tested for changes in giant fiber conduction velocity, the latter being a reliable indicator of nervous system growth (O'Gara et al., 1982). As shown in Figure 5, Cd treatment caused a dose-dependent stunting of somatic growth; at the highest concentration (500 ppm) weights were less than one-tenth those in the control group. In contrast to the effects of Cd on body growth, there was little or no effect on giant fiber growth, except at the highest concentration (Fig. 6). These results clearly indicate that the nervous system was spared with respect to the growth-stunting effects of Cd.

How could Cd stunt somatic growth? One possibility is that Cd somehow causes reduced food consumption, for example by decreasing microbial food content or by causing distastefulness of the food source. One way to test this possibility is to examine somatic and nervous system growth under conditions of decreased food availability (i.e., starvation). Vining et al. (1982) studied the effects of starvation on somatic and nervous system growth during postembryonic development in Eisenia foetida. Their results showed a dose-dependent stunting of body growth essentially identical to that seen with Cd treatment. As with Cd treatment, stunting of giant fiber growth, as indicated by low conduction velocities, occurred only during the most severe food deprivation and were readily reversed by replenishing food. Thus the effects of

Cd treatment were virtually indistinguishable from those of starvation, suggesting that Cd somehow caused a reduction in food consumption.

CLASS SPECIFICITY OF NEUROPHYSIOLOGICAL AND BEHAVIORAL EFFECTS

Pesticides can be classified in various groups depending on their general chemical configuration (e.g., organophosphates, carbamates, organochorines, etc.). Common features of chemical structure within a group often result in common modes of toxic action and symptomology. Conversely, differences in structure between groups may be associated with different modes of action and symptomology.

Although we have not yet examined the neurotoxic effects of many pesticides, some preliminary results shown in Table 1 suggest that there may be some class differences with respect to sublethal neurophysiological and behavioral symptoms. These may indicate differences in the fundamental mechanisms of action for each class of pesticide. There is clearly a need for more comprehensive study of this question.

In conclusion, the possibilities of using earthworms as sensitive indicators for ecotoxicity testing appear promising. For the purposes of assessing sublethal neurophysiological effects, the methods we have described herein may be particularly useful. On the other hand, for assessment of other toxicity effects in earthworms, tests involving biochemical assay (Stenersen, 1979), growth (Lofs-Holmin, 1980; Karnak and Hamelink, 1982), or reproductive capacity (Lofs-Holmin, 1982) may be preferable.

ACKNOWLEDGEMENTS

We thank Dr. E. Neuhauser for many helpful suggestions, Dr. J. Somers-Reed for providing samples of Diazinon, B. O'Gara for technical assistance, and M. Nims for typing the manuscript. This research was funded, in part, by the Hazardous Materials Assessment Program of the United States Environmental Protection Agency (EPA Cooperative Agreement CR8100060-01). It was administered by the Corvallis Environmental Research Laboratory as part of a research program for evaluating the effectiveness of various bioassays in determination of the biological impact of various hazardous materials.

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FIGURE LEGENDS

Fig. 1. Calculation of conduction velocity for giant nerve fiber impulses. Four electrode pairs in contact with the ventral surface of a worm (Lumbricus terrestris) were used to obtain the four-channel oscilloscope recording shown at the right. A single, anteriorly conducted lateral giant fiber impulse was evoked by very light tactile stimulation to the tail.

Fig. 2. Toxicity effects in Eisenia foetida following 48 h contact exposure to dieldrin. Mortality is shown on the right coordinate, each "x" indicating % mortality for a group of 10 worms. Relative conduction velocity is shown on the left coordinate, each dot indicating the mean ± SEM (n=6 animals). Note the sublethal reductions in relative velocity of medial (dots) and lateral (circles) giant nerve fibers. Lower case letters indicate statistical significance: (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$. (Modified from Drewes et al., 1984).

Fig. 3. Conduction velocity effects in Eisenia foetida following two-week artificial soil exposure to dimethyl phthalate. Relative conduction velocity values in both medial (dots) and lateral (circles) giant nerve fibers were significantly reduced by treatments. Each point indicates a mean ± SEM ($n = 6$ animals). Lower case letters indicate statistical significance: (a) $p < 0.01$; (b) $p < 0.001$.

Fig. 4. Conduction velocity effects in Lumbricus terrestris following a two-minute immersion exposure to Diazinon. Points indicate mean relative velocity ± SEM ($n = 6$ animals) for the medial giant nerve fiber. Dose-dependent decrease in velocity were evident, with approximately one-half of the total reduction occurring in the first 15 min following treatment.

Worms at the highest concentration were dead by 2 h, but worms in the other groups recovered.

Fig. 5. Effects of Cd treatment on postembryonic growth in Eisenia foetida. Each point represents the mean weight (± SD) of six worms. A dose-dependent stunting of body growth was evident.

Fig. 6. Effects of Cd treatment on postembryonic development of medial giant fiber conduction velocity in Eisenia foetida. At the two lowest concentrations velocity increases were essentially identical to those in the control group. At the highest concentration, velocity was reduced in comparison to the control group. Each point represents the mean ± SEM (n = 6 animals). These data are from the same worms shown in Figure 5.

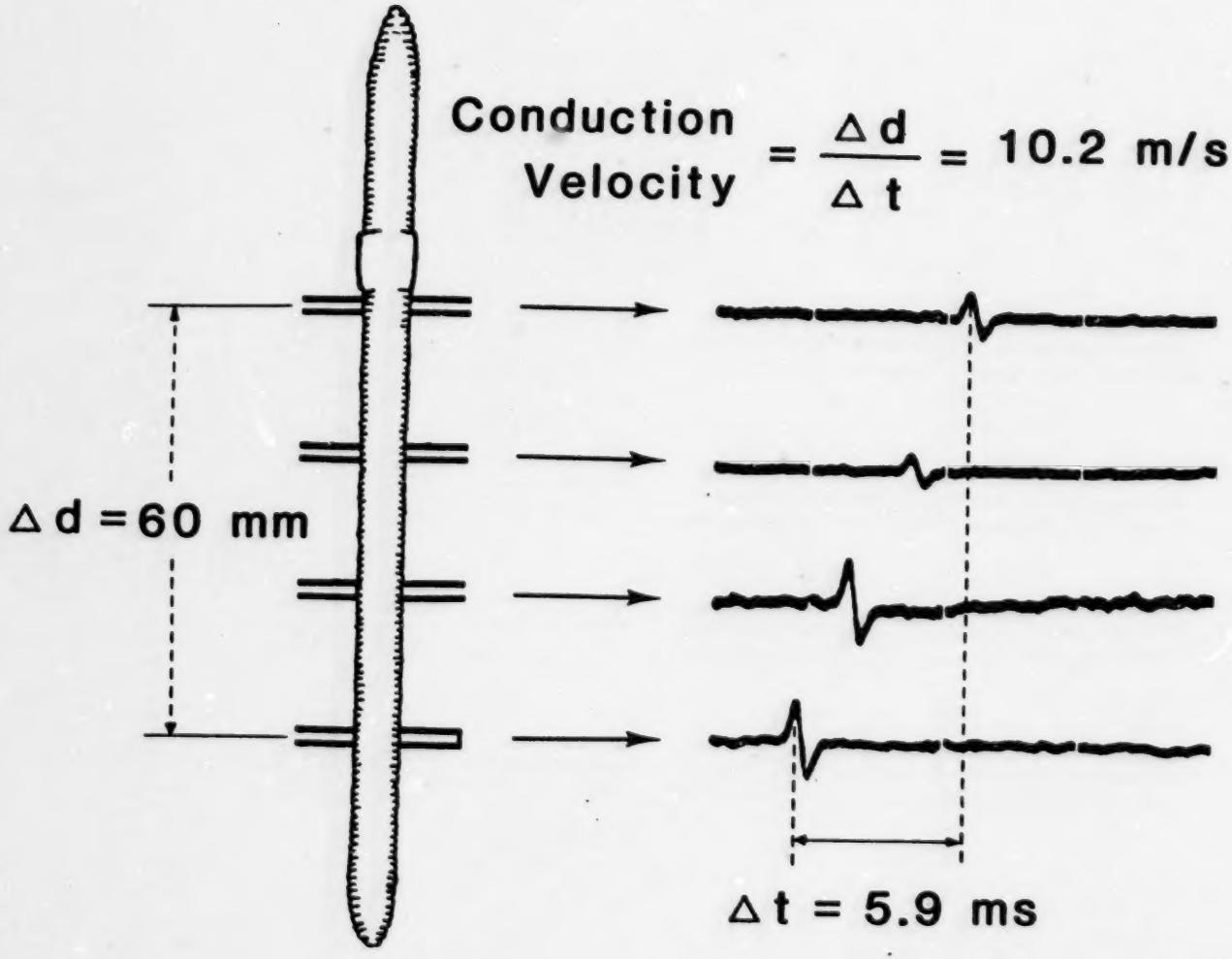

Fig. 1

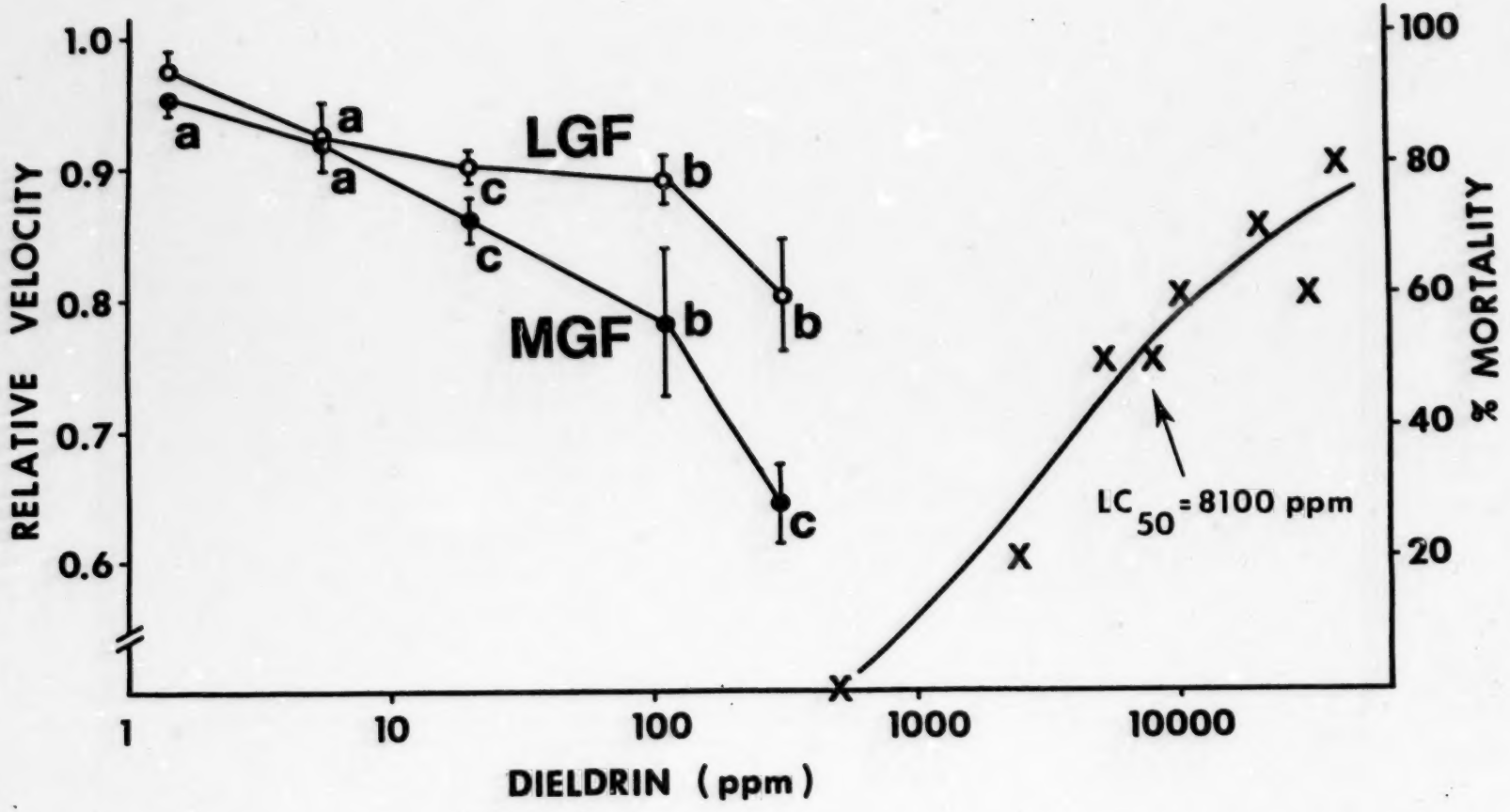
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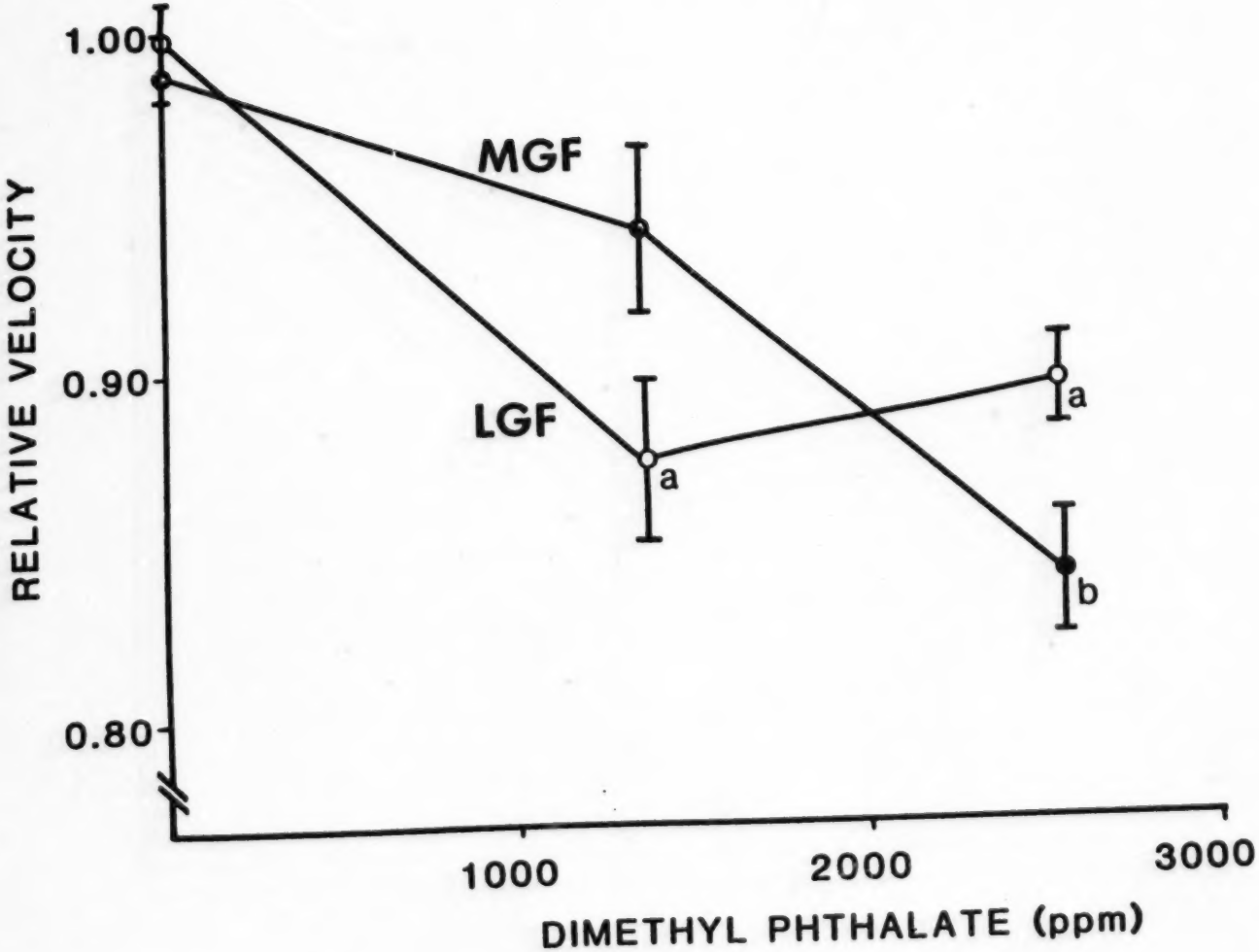
Fig. 2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DIMETHYL PHTHALATE (ppm)

Fig. 3

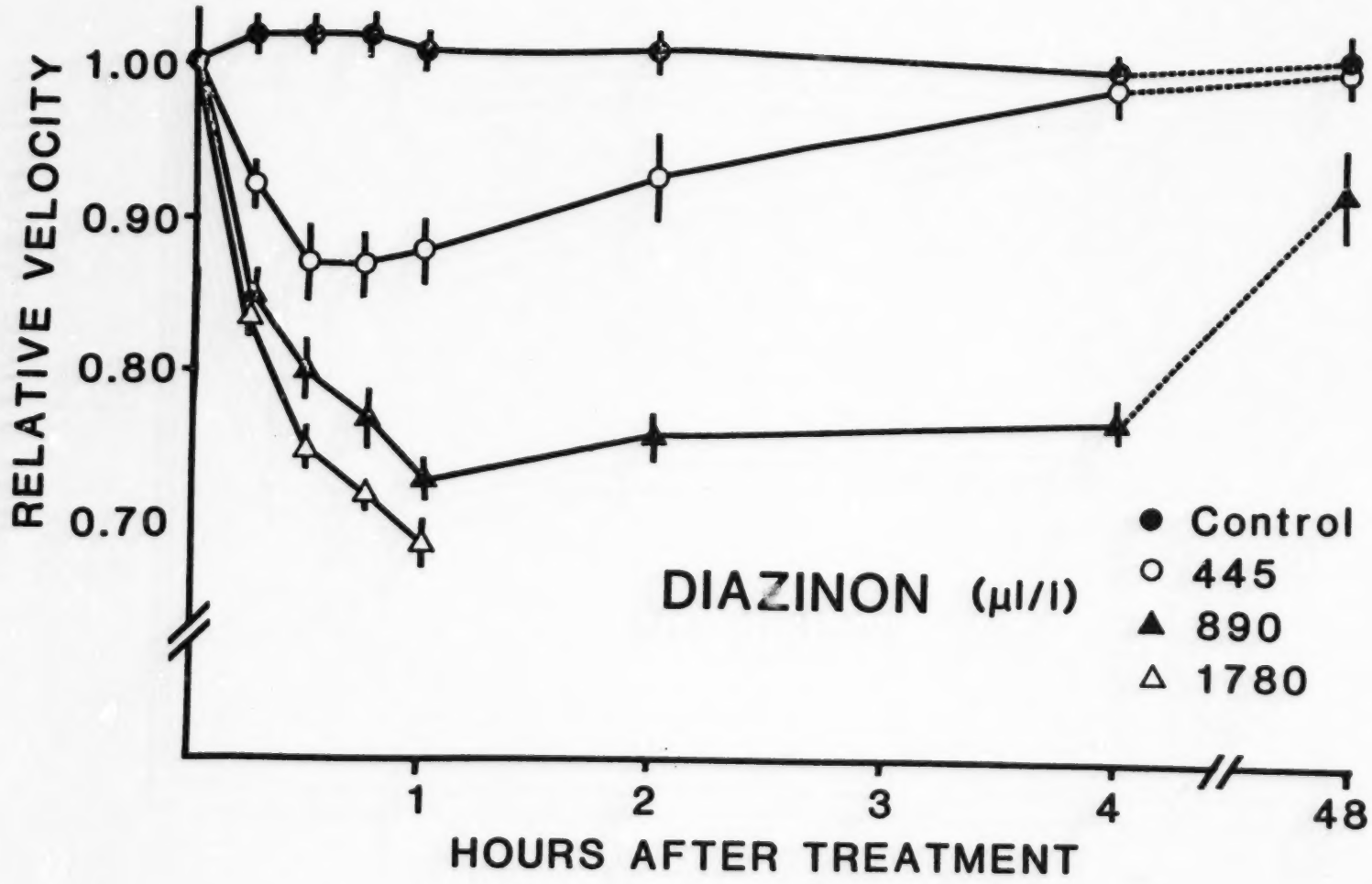
RELATIVE VELOCITY
1.00
0.90
0.80
0.70
DIAZINON (µl/l)
Control
445
890
1780
1
2
3
4
48
HOURS AFTER TREATMENT

Fig. 4

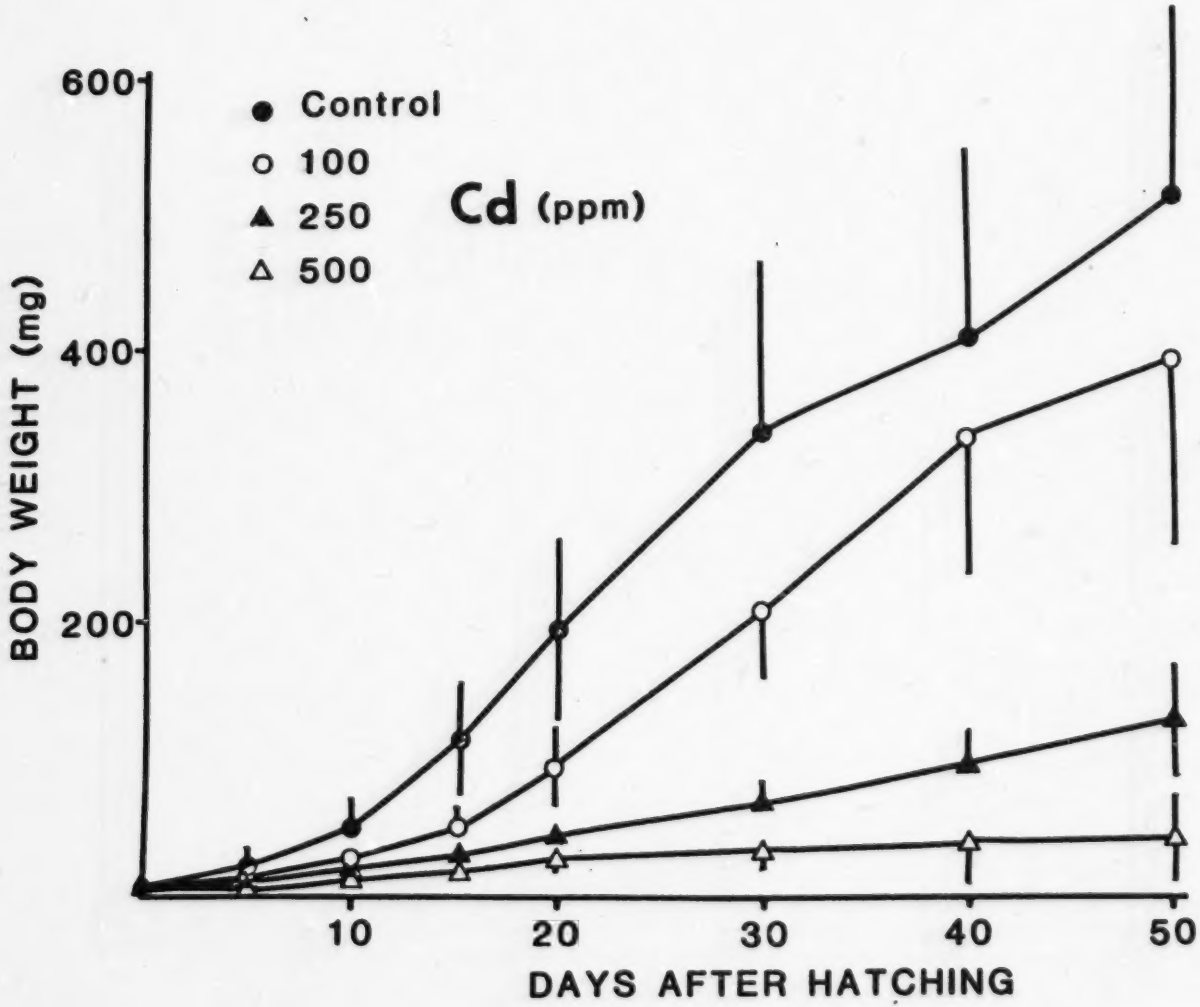
600
400
200
BODY WEIGHT (mg)
Control
100
250
500
Cd (ppm)
10
20
30
40
50
DAYS AFTER HATCHING

Fig. 5

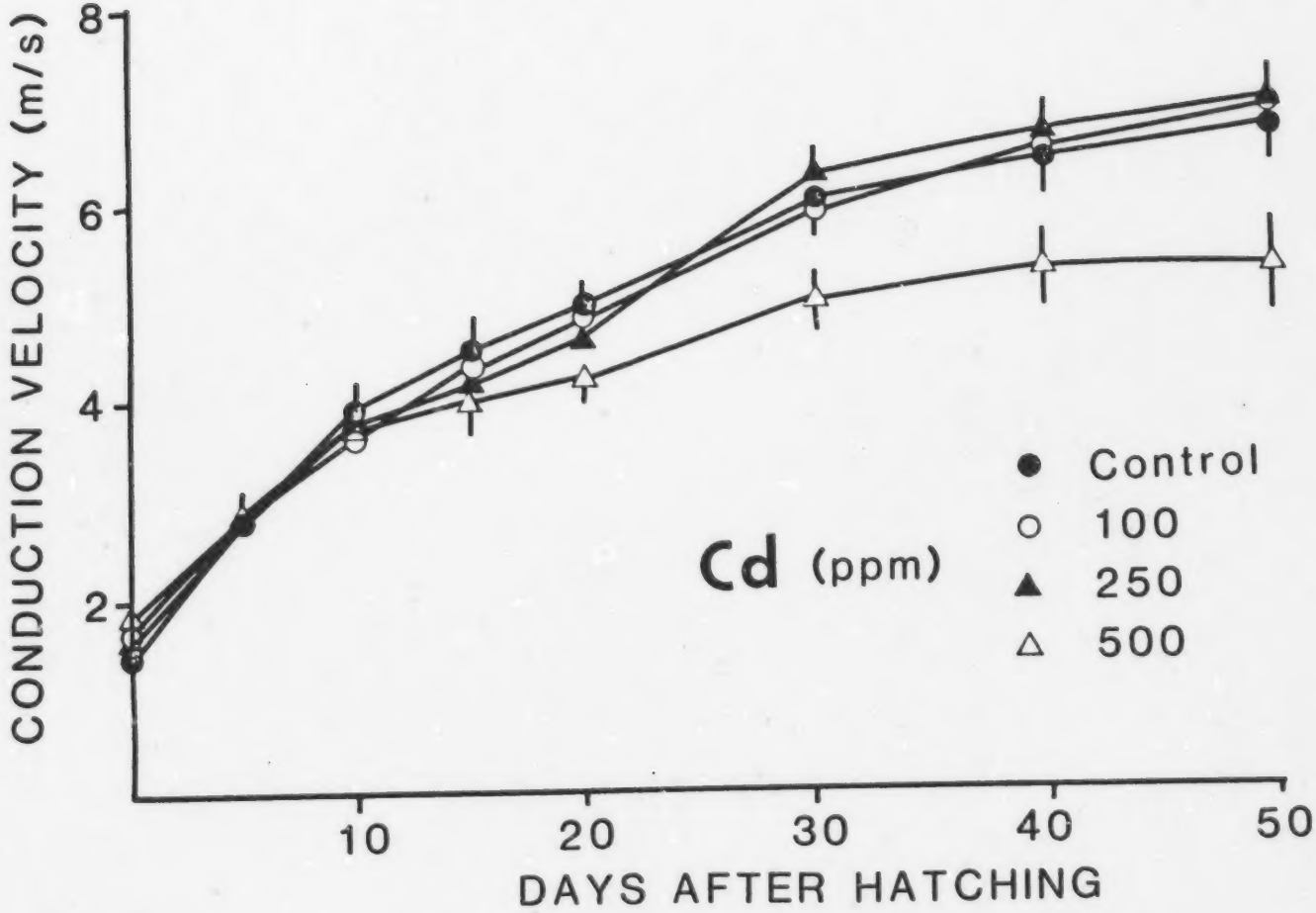
CONDUCTION VELOCITY (m/s)
8
6
4
2
10
20
30
40
50
DAYS AFTER HATCHING
Cd (ppm)
Control
100
250
500

Fig. 6

Table 1. Sublethal effects following 48 h contact tests in Eisenia foetida

Neurophysiological symptoms	CARBAMATE Carbaryl (2.5 ppm)	ORGANOCHLORINE Dieldrin (5 ppm)	ORGANOPHOSPHATE Diazinon (0.1 µl/ml water)	PYRETHROID Fenvalerate (15.5 ppm)
(1) MGF conduction velocity	OD	SD	OD	N
LGF conduction velocity	OD	SD	OD	N
SD-significant decrease OD-occasional decrease N-no change				
(2) Touch sensitivity	N	HH	OH	N
HH-highly hypersensitive OH-occasional hypersensitivity N-normal sensitivity				
(3) MGF-mediated muscle potentials	N	D	N/D	N
D-decreased N-no effect				
Behavioral/overt symptoms				
(4) Peristaltic locomotion	W/A	W/A	W/A	N
A-ataxic, W-weak, N-normal				
(5) Other behavioral signs	C	S	C	W
S-shortening W-writhing C-coiling				
(6) Lesions (see Roberts and Dorough, 1984)	MSS	-	B	-
MSS-mid segmental swelling B-bleeding from ends				